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学位授与の題目	Biochemical Studies on Ferredoxin-NADP+Oxidoreductase(FNR) and Thermostable Glucosidase in Cyanobacteria.（シアノバクテリアにおけるフェレドキシンーNADP＋酸化還元酵素および耐熱性グルコシダーゼの生化学的研究）
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Abstract

Ferredoxin-$NADP^+$ oxidoreductase (FNR) catalyzing the terminal step of the linear photosynthetic electron transport was purified to homogeneity from the cyanobacterium *Spirulina platensis* and the red alga *Cyanidium caldarium.* FNR of *Spirulina* consisted of three domains (CpcD-like domain, FAD-binding domain and $NADP^+$-binding domain) with a molecular mass of 46 kDa and was localized in either phycobilisomes or thylakoid membranes. The membrane-bound FNR with 46 kDa was solublized by NaCl and the solublized FNR had an apparent molecular mass of 90 kDa. FNR of *Cyanidium* consisted of two domains (FAD-binding domain and $NADP^+$-binding domain) with a molecular mass of 33 kDa. In *Cyanidium*, FNR was found on thylakoid membranes but there was no FNR on phycobilisomes. The membrane-bound FNR of *Cyanidium* was not solublized by NaCl, suggesting the enzyme is tightly bound in the membrane.

A thermostable beta D-glucosidase was purified to homogeneity from the cyanobacterium *Nostoc commune*. The molecular mass of the purified protein was 20 kDa and its N-terminal amino-acid sequence was identical with the cyanobacterial secreted and surface fasciclin domain proteins. The beta D-glucosidase had a neutral optimum pH value of 7 and showed a lower affinity towards other sugars.

Comparative Study of Ferredoxin $NADP^+$ Oxidoreductase in Cyanobacteria and Red Algae.

Cyanobacterial FNR in *S. platensis.*

FNR was purified from *S. platensis* and was electrophoretically homogeneous with a molecular mass of 46 kDa. The N-terminal amino-acid sequence of the purified protein was [MYSPTGTGVAMR] and identical to the deduced amino-acid sequence from the *petH* gene of *S. platensis*. The 46 kDa FNR of *S. platensis* degraded *in vitro* at room temperature in the absence of protease inhibitors to form the 34 kDa FNR. During the incubation of the crude extract at room temperature, the FNR proteins with intermediate size of 37 kDa, 36 kDa, 35 kDa and 34 kDa, respectively, were produced in 2 h, and these cleavages of FNR protein specifically took place in the hinge region, which is the interspace region between the CpcD-like domain and the catalytic domains, according to the N-terminal amino-acid sequences of these intermediate size FNRs

(Figure 1). Because the 34 kDa FNR showed high diaphorase activity similarly to the 46 kDa FNR, the truncation of the CpcD-like domain did not affect the activity of FNR of *S. platensis.*

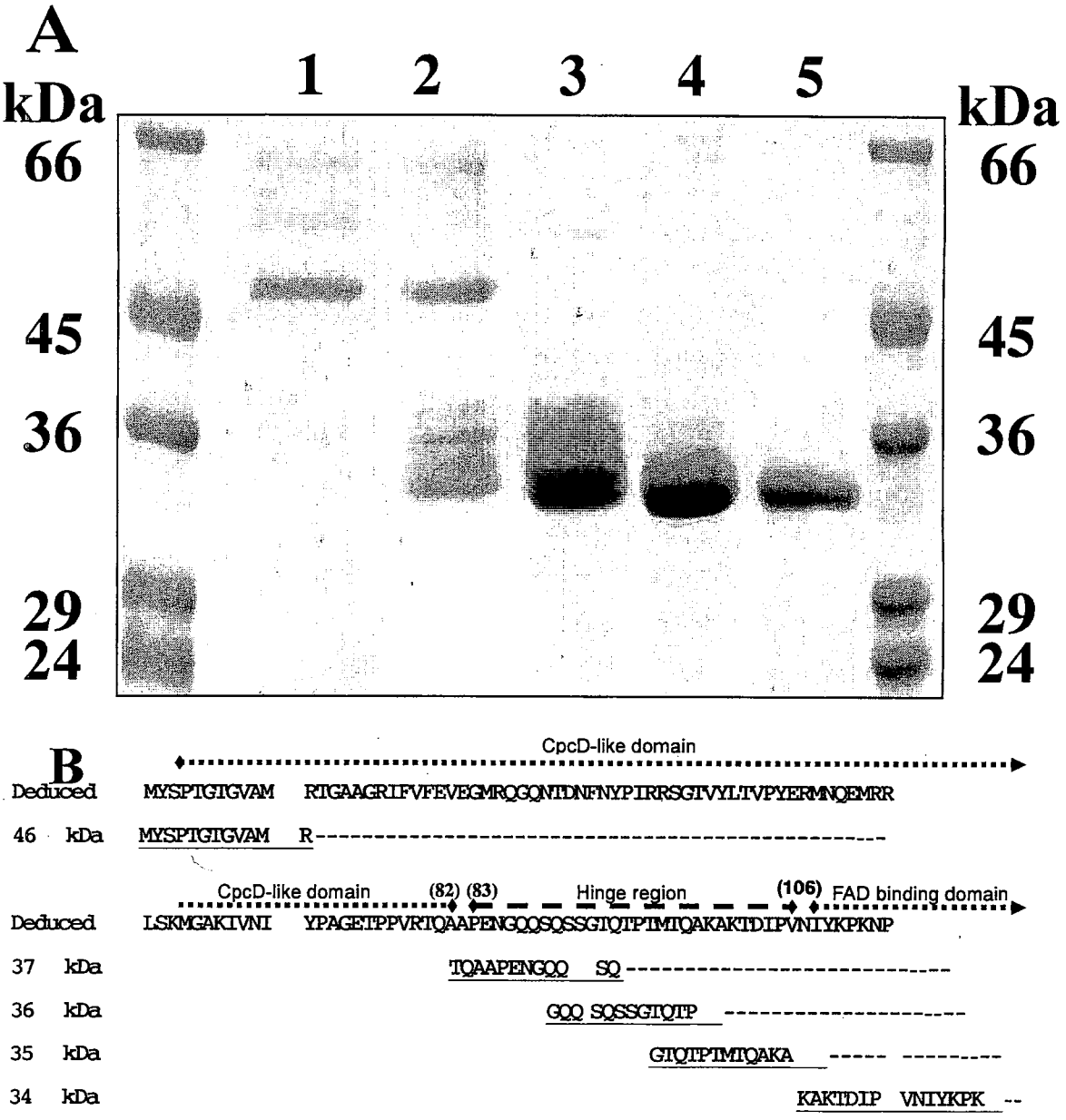

Figure (1): Site specific degradation of the *S. platensis* 46 kDa FNR *in vitro*. (A) The 46 kDa FNR purified in the presence of protease inhibitors (lane 1) and intermediate size FNRs purified after the incubation at room temperature for 1 h (lane 2), 2 h (lane 3), 3 h (lane 4) and 4 h (lane 5) were fractionated by SDS-PAGE and stained by CBB. FNR of 100 mU of DCPIP reduction activity was loaded on each lane. A molecular size marker (Dalton mark VII-L, Sigma, SDS-7) was used for the estimation of the molecular size. (B) The N-terminal amino-acid sequences of the bands in a range of 34 to 37 kDa were determined by automated Edman degradation and aligned with the deduced amino-acid sequence of the *petH* gene (accession no. AB113346).

Red-algal FNR in *C. caldarium*.

The *C. caldarium* FNR was purified to homogeneity with a purification factor of 554 fold. The purified FNR was electrophoretically homogeneous with an apparent molecular mass of 33 kDa. The N-terminal amino-acid sequence of the FNR of *C. caldarium* was [VAAEKKVP], which was identical to the deduced amino-acid sequence from the cDNA encoding FNR of *C. caldarium*.

Subcellular localization of FNR in *S. platensis* and *C. caldarium*.

FNR activity on phycobilisomes isolated from *S. platensis* was analyzed. The phycobilisomes isolated from *S. platensis* contained a 46 kDa protein, which was identified to be FNR by N-terminal sequencing.

The FNR bound on thylakoid membranes of *S. platensis* was solubilized using 1 M NaCl. No FNR activity was detected in thylakoid membranes of *S. platensis* after solubilization by NaCl, suggesting that FNR was extrinsic electrostatically solubilized by NaCl. The 46 kDa FNR was released from the thylakoid membranes and no 34 kDa FNR was detected on the thylakoid membranes. On a gel filtration, the NaCl solubilized FNR from *S. platensis* thylakoid membranes had an apparent molecular mass of 90 kDa and contained the 46 kDa FNR and a 17 kDa hypothetical protein. No detectable FNR activity was found in phycobilisomes isolated from the red alga *C. caldarium*, suggesting that phycobilisomes of *C. caldarium* did not contain FNR. Approximately 32 % of the total FNR molecules was bound to thylakoid membranes of *C. caldarium* and approximately 68% of FNR was soluble in the cell free extract. The thylakoid membranes-bound FNR of *C. caldarium* was not solubilized by 1 M NaCl, indicating that the FNR was tightly bound to the thylakoid membranes of *C. caldarium.*

Comparison of biochemical characteristics of the purified FNR.

In the ferredoxin-dependent cytochrome C reduction activity of FNR, the activities decreased by increasing the pH value and the optimum pH values for all forms of FNR were pH 7. In contrast of the pH dependency of the ferredoxin-dependent activity, the quinone-dependent cytochrome C reduction activity of FNR increased by increasing the pH value and the optimum pH values for all forms of FNR were 9.7. The ferredoxin-dependent cytochrome C reduction activity decreased sharply as the ionic strength of the reaction medium increased. But the quinone-dependent cytochrome C reduction activity increased with increasing the ionic strength of the reaction medium and the optimum ionic strength was 300 mM NaCl for all forms of FNR. These results suggest that both phycobilisome-bound and thylakoid-bound FNR function as a quinone reductase similarly as ferredoxin-$NADP^+$ oxidoreductase.

Characterization of extacellular fasciclin domain protein with glucosidase activity.

A new method for massive extraction of the extracelluar polysaccharides (EPS) from cells of *N. commune* colonies was developed in this study. The EPS of *N. commune* was massively extracted after stirring overnight in the high concentration 0.75 M potassium phosphate buffer (pH 7) followed by homogenization. Two distinct layers

were distinguished; the upper EPS layer and the lower aqueous green layer containing the EPS-depleted cells. The EPS-depleted cells of *N. commune* represented 4.7 % of the total fresh mass of *N. commune* colonies indicating that around 95 % of the total fresh mass of *N. commune* were in the water hydrated EPS.

The cyanobacterium *N. commune* contained several kinds of glycosidases activities. Most of the gylcosidases activities were detected in the water soluble fraction retained in the 0.75 M phosphate buffer, pH 7. Of the glycosidases activities in *N. commune*, only a thermostable beta D-glucosidase was detected after boiling the crude homogenate of the *N. commune* colonies. The water soluble fraction retained in the 0.75 M potassium phosphate buffer contained 97 % of the total thermostable beta D-glucosidase activity of *N. commune*. Such thermostable beta D-glucosidase was purified to homogeneity with a purification factor of 607 fold and showed a single band on SDS PAGE, The N-terminal amino acid sequence of the purified 20 kDa thermostable beta D-glucosidase was (MNIVDTAVNNGSFNTLVAAI) and identical with the surface and secreted fasciclin domain protein from the cyanobacteria. The protein specifically hydrolyzed beta-D-glucosides but showed low affinity towards other beta- or alpha-linked glycosides. The optimum pH of the beta-D-glucosidase activity of the purified fasciclin domain protein was pH 7. The purified fasciclin domain protein was thermostable in its beta-D-glucosidase activity as its activity was tolerant to boiling for 20 min.

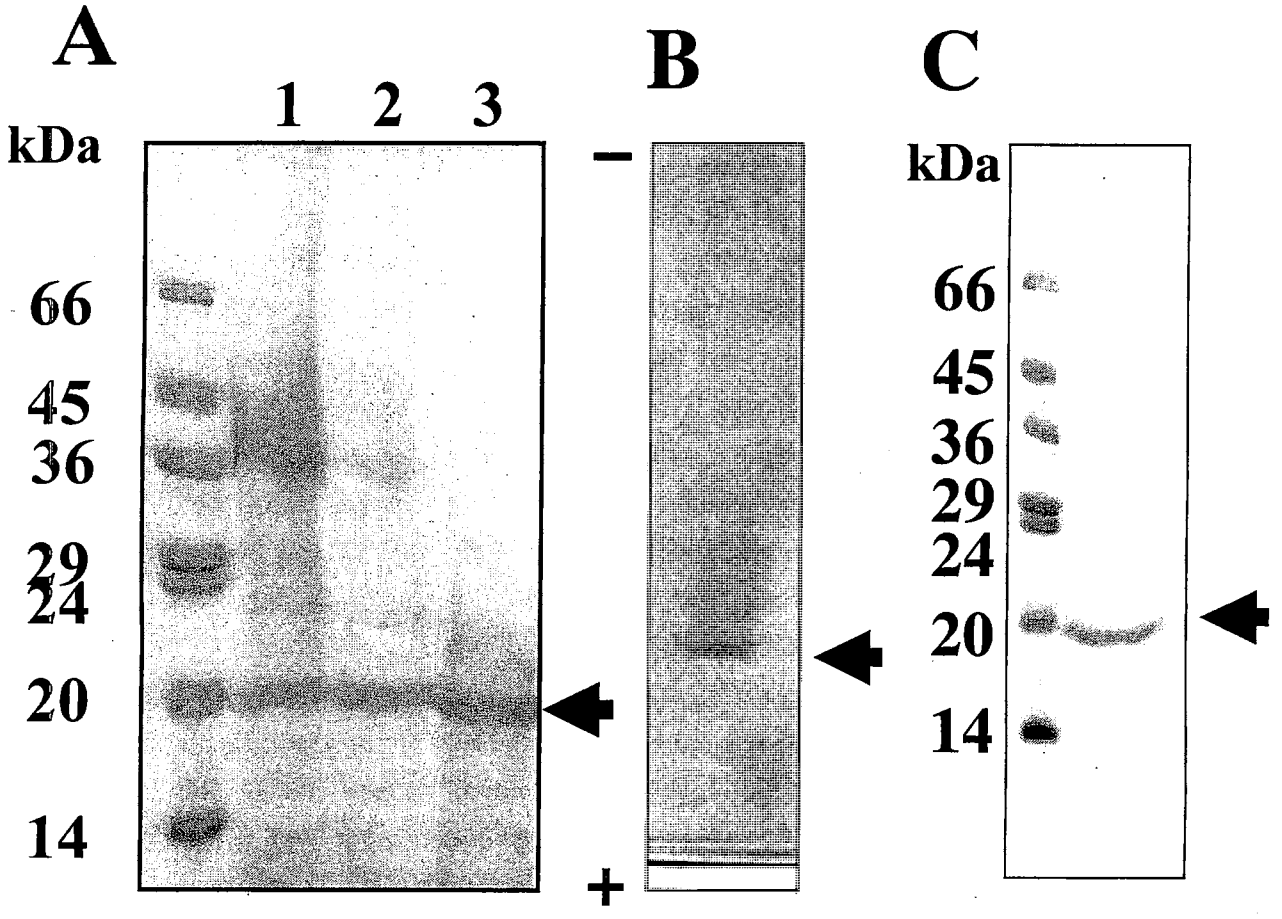

Figure (2): The thermostable β-D-glucosidase from the terrestrial cyanobacterium *Nostoc commune*. (A) Purification of the thermostable β-D-glucosidase from *N. commune*. The water soluble fraction solubilized by the 0.75 M phosphate buffer containing 100 mU of the thermostable β-D-glucosidase activity (lane 1), 40-80 % ammonium sulfate precipitation fraction containing 100 mU of the thermostable β-D-glucosidase activity (lane 2) and active fraction after DEAE-cellulose chromatography containing 200 mU of the thermostable β-D-glucosidase activity (lane 3) were fractionated by SDS-PAGE and stained by CBB.. The arrow head shows the thermostable β-D-glucosidase band. (B) The purified thermostable β-D-glucosidase (100 mU) was fractionated by PAGE under non-denaturing conditions and stained by CBB. The arrow head shows the single native band of the β-D-glucosidase. (C) The native band of the β-D-glucosidase eluted from a corresponding three lanes from the native gel had 260 mU activity and was fractionated using SDS-PAGE and stained by CBB. A molecular size marker (Dalton mark VII-L, Sigma, SDS-7) was used for a standard.

学位論文審査結果の要旨

本論文は，光合成電子伝達の最終段階で NADPH の合成を触媒する酵素であるフェレドキシン-NADP+酸化還元酵素に関して集光性アンテナとしてフィコビリソームもつラン藻スピルリナおよび紅藻シアニジウムにおいて行なった比較解析と陸棲ラン藻において見いだされた耐熱性β-グルコシダーゼに関する生化学的解析について記載している。

ラン藻スピルリナのフェレドキシン-NADP+酸化還元酵素はアミノ末端にフィコビリソームに会合するためのドメインをもち，フィコビリソームに結合していた。これに対して，紅藻シアニジウムのフェレドキシン-NADP+酸化還元酵素はフィコビリソームに結合するためのドメインをもたず，単離したフィコビリソーム中に全く酵素活性が検出されなかった。本酵素がフィコビリソームに結合しているという性質はラン藻に限定して見られる特徴であることが明らかとなった。この成果は国際光合成学会の学会誌である Photosynthesis Research に掲載されることが決定している。続いて陸棲ラン藻の耐熱性β-グルコシダーゼを精製して生化学的解析を行なった。本酵素は分子量およそ 20kDa の分泌性のタンパク質でありことが分かり，細胞外マトリックスに局在し機能が不明確であったンパク質の役割の一部を解明することができた。

以上の研究成果は当該学問分野における新知見を含むものであり，審査委員会として博士（理学）に値すると判断した。